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Lymphocyte subset differences in patients with chronic fatigue syndrome, multiple sclerosis and major depression

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Summary

Chronic fatigue syndrome (CFS) is a heterogeneous disorder of unknown aetiology characterized by debilitating fatigue, along with other symptoms, for at least 6 months. Many studies demonstrate probable involvement of the central and autonomic nervous system, as well as a state of generalized immune activation and selective immune dysfunction in patients with CFS. The aim of this study was to compare the lymphocyte subsets of patients with chronic fatigue syndrome to those of patients with major depression and multiple sclerosis as well as those of healthy control subjects. No differences were found in total numbers of T cells, B cells or natural killer (NK) cells. However, differences were found in T, B and NK cell subsets. Patients with major depression had significantly fewer resting T ($CD3^+/CD25^-$) cells than the other groups. Patients with major depression also had significantly more $CD20^+/CD5^+$ B cells, a subset associated with the production of autoantibodies. Compared to patients with multiple sclerosis, patients with CFS had greater numbers of $CD16^+/CD3^-$ NK cells. Further study will be required to determine whether these alterations in lymphocyte subsets are directly involved in the pathophysiology of these disorders, or are secondary effects of the causal agent(s).

Keywords: B cell, chronic fatigue syndrome, depression, NK cell, multiple sclerosis, T cell

Introduction

Chronic fatigue syndrome (CFS) is a heterogeneous disorder of unknown aetiology characterized by debilitating fatigue, myalgia, arthralgia, headache, sore throat, adenopathy, sleep disruption, difficulties with memory and concentration and post-exertional malaise [1,2]. Because of its clinical features and frequent onset after an acute febrile illness, one commonly held hypothesis is that CFS is due to infection with one or more viruses [3], leading to chronic, low-level activation of the immune system characterized by the overproduction of several cytokines. This immunological activation, in turn, is postulated to alter neurotransmitter function and to lead to the complex of symptoms seen in CFS. Although a number of studies have associated various infectious agents with CFS [4–10] most investigators do not believe that a single novel infectious agent will be shown to cause CFS. Indeed, no known agent has been conclusively shown to cause CFS.

Many studies have demonstrated central and autonomic nervous system abnormalities in patients with CFS, including neuroendocrine abnormalities reflecting dysfunction of the hypothalamus and pituitary gland [11–32]. Another commonly held hypothesis is that these neuroendocrine abnormalities are the primary disorder, and that they lead to secondary immune dysfunction. A variant of this hypothesis is that a chronic low-grade central nervous system infection triggers the neurological abnormalities seen in CFS.

Numerous immunological abnormalities have been described in patients with CFS. While a number of these putative abnormalities have been inconsistently reported, a few appear to be more robust: depressed natural killer (NK) cell function by *in vitro* testing, increased numbers of activated cytotoxic T cells, increased levels or production of proinflammatory cytokines, and deficiencies in immunoglobulin subclasses [33]. However, past studies have not included comparison groups with other fatiguing illnesses.

The aim of this study was to compare lymphocyte subsets in patients with CFS, major depression and multiple sclerosis (MS) and healthy control subjects. Patients with major depression and MS often experience symptoms of chronic

fatigue in the course of their illness. We hypothesized that if the immunological abnormalities observed in CFS were specific and relevant to its pathogenesis, then immunological parameters in CFS should differ from those of disorders that share some of its symptoms but have a different pathophysiology.

Methods

Subject selection

Patients with CFS were recruited from those seen in the practice of A.L.K. and met the 1988 Centers for Disease Control and Prevention (CDC) case definition for CFS [34], which was the current definition at the time this study was initiated. Under this case definition there are 11 symptom criteria (fever, sore throat, painful lymph nodes, weakness, myalgia, post-exertional fatigue, headaches, arthralgia without redness or swelling, neuropsychological complaints, sleep disturbance and sudden onset) and three physical examination criteria (low-grade fever, non-exudative pharyngitis and cervical or axillary adenopathy). The physical examination criteria must be documented by a physician on two occasions at least 1 month apart. To establish a diagnosis of CFS under this definition, a patient must have a new onset of persistent or relapsing fatigue that reduces average daily activity by 50% for at least 6 months and exhibit either (a) six symptom and two physical examination criteria, or (b) eight symptom criteria. Other clinical conditions that could cause similar symptoms must be excluded. The patients with CFS that were selected for this study were not chosen because of the intensity of their illness symptoms: the only requirement was that they met the then-current CDC criteria. Patients were receiving symptomatic treatments at the time of the study.

Patients with major unipolar depression at the time of recruitment were enrolled from the practices of K.L.B. and S.N.W. All subjects were freely consenting out-patients, and were included if they met criteria for major depression as indicated in the *Diagnostic and Statistical Manual for Psychiatry* version III, revised (DSM-III-R). All were receiving standard treatments for depression, including pharmacological agents.

Patients with MS were recruited from the practice of G.A.M. from a large university MS speciality clinic. All patients had relapsing-remitting MS and met the criteria set forth by Poser and colleagues [35], including strongly confirmatory brain magnetic resonance imaging (MRI) scans. All enrolled patients had Kurtzke expanded disability status scores (EDSS) of 1·0 (no disability, minimal neurological signs) to 3·0 (moderate disability, fully ambulatory), and Hauser ambulation index scores of 1 (normal gait, significant fatigue) or 2 (noticeably abnormal gait or episodic imbalance). None had co-morbid conditions that could have contributed to their fatigue. In the MS patients selected for study, fatigue limited their activities by more than 50%, constituted their major MS-related limiting symptom, and was clearly disproportionate to their EDSS neurological impairments. None was clinically depressed or was taking antidepressant medication. None had ever received myelosuppressive or immunomodulatory treatment. None had had an MS attack or had received corticosteroids within 30 days prior to study entry and phlebotomy.

Control subjects were recruited from hospital employees who were asked to complete a health questionnaire to ensure that they were in good general health and had no history of depression, MS, CFS or any of the chronic symptoms that are required for a diagnosis of CFS. This study was approved by the Institutional Review Boards of Brigham and Women's Hospital, Boston, MA and the former New England Deaconess Hospital (now Beth Israel Deaconess Medical Center), Boston, MA. Written informed consent was obtained from all subjects prior to collecting blood samples.

At the time of enrolment, subjects in the depression, MS and control groups were matched for age (within 5 years) and sex to the patients with CFS. All blood samples were drawn between 8 and 11 o'clock in the morning to reduce variations caused by the normal daily fluctuations in lymphocyte subset levels.

Antibodies and reagents

Fluorochrome-conjugated murine monoclonal antibodies obtained from Coulter Immunology (Hialeah, FL, USA) included T11 (CD2), T3 (CD3), T4 (CD4), T1 (CD5), T8 (CD8), Mo1 (CD11b), 3G8 (CD16), B1 (CD20), B6 (CD23), IL-2R1 (CD25), 4B4 (CD29), NKH1 (CD56) and I-2 (HLA-DR). Fluorochrome-conjugated Leu-7 (CD57) was purchased from Becton-Dickinson (Mountain View, CA, USA). Fluoroscein isothiocyanate (FITC)-conjugated HB-7 (CD38) was a kind gift of Dr Thomas Tedder (Duke University Medical Center, Durham, NC, USA).

Isolation of peripheral blood mononuclear cells (PBMC)

Approximately 120 ml of fresh whole blood were collected in tubes containing ethylenediamine tetra-acetic acid (EDTA). Blood samples were coded prior to delivery to the laboratory, so that personnel performing the laboratory tests were blinded to the identity of the subjects. Peripheral blood mononuclear cells (PBMC) were separated by Ficoll-diatrizoate density gradient centrifugation, washed in medium containing 5% human antibody serum and cryopreserved in liquid nitrogen.

Immunofluorescence analysis and calculation of lymphocyte subset numbers

Cryopreserved PBMC were thawed, washed in medium containing 5% human AB serum, stained directly with FITC-

Table 1. Absolute numbers of total lymphocytes and major lymphocyte subsets.[a]

Subset	CFS	Depression	MS	Control	*P*-value[b]
Total lymphocytes	1900 (23)	2095 (24)	2130 (22)	1760 (25)	0·499
$CD2^+$ PBL[c]	1172 (23)	944 (24)	1228 (22)	1197 (25)	0·156
$CD3^+$ T cells	940 (23)	816 (24)	1097 (22)	974 (25)	0·352
$CD20^+$ B cells	348 (23)	432 (23)	272 (21)	245 (23)	0·048
$CD56^+$ NK cells	284 (23)	230 (24)	216 (22)	201 (25)	0·441

[a]Values are median absolute number of cells per μl. Numbers in parentheses indicate the number of samples tested. [b]P-values from Kruskal–Wallis ANOVA on Spearman's rank test. None of the differences is significant using a level of 0·01. [c]Includes virtually all T cells and the majority of natural killer (NK) cells.

and phycoerythrin (PE)-conjugated monoclonal antibodies, and incubated at 4°C in the dark for 20–30 min; the cells were then washed, fixed in 1% formaldehyde in phosphate-buffered saline (PBS), and stored in the dark at 4°C until flow cytometric analysis. Two-colour flow cytometry was performed using an EPICS 752 or EPICS Elite instrument from Coulter (Hialeah, FL, USA); 10 000 events were collected for each sample. During analysis, forward- and side-scattering properties were used to create a lymphocyte gate. Thresholds for discriminating levels of staining above background were established by analysis of PBMC stained with FITC- and PE-conjugated control monoclonal antibodies. The absolute number of various lymphocyte subsets was calculated by multiplying the total lymphocyte count (derived from a routine complete blood count performed on the same day a blood sample was obtained) by the percentage of cells in a sample expressing the relevant phenotype (derived from flow cytometric analysis).

The lymphocyte subsets analysed in this study are summarized in Tables 1 and 2. Data from other investigators on immunological abnormalities in patients with CFS were presented and/or published during the course of this study and surface antigens tested by immunophenotyping were modified accordingly. Therefore, not every blood sample was tested for every lymphocyte subset.

Statistical analysis

For each sample the number of $CD2^+$ cells was derived from single-colour analysis. The absolute numbers of other major lymphocyte subsets (i.e. $CD3^+$, $CD8^+$, $CD20^+$, $CD56^+$) were calculated from the results of two-colour staining. For

Table 2. Lymphocyte subsets with significant differences between subject groups.

Subset	CFS	Depression	MS	Healthy controls	Four-way *P*-value	Pairwise statistically significant differences	Trends (not statistically significant)
T cell subsets							
$CD3^+/CD25^-$	892 (14)	376 (11)	809 (13)	1009 (12)	0·006*	Dep ↓ *versus* All	
$CD3^+/CD16^+$	169 (14)	172 (11)	88 (13)	72 (12)	0·259		CFS ↑ *versus* controls
							Dep ↑ *versus* controls
$CD3^+/CD25^+$	121 (14)	155 (11)	74 (13)	81 (12)	0·061		Dep ↑ *versus* MS
$CD8^+$/HLA-DR^+	64 (23)	34 (23)	47 (22)	26 (24)	0·082		CFS ↑ *versus* controls
$CD8^+/CD38^+$	196 (18)	133 (24)	90 (22)	134 (23)	0·057		CFS ↑ *versus* MS
B cell subsets							
$CD20^+/CD5^+$	130 (19)	471 (11)	100 (11)	154 (14)	0·004*	Dep ↑ *versus* All	
$CD20^+/CD25^+$	62 (19)	89 (11)	29 (13)	30 (14)	0·044		CFS ↑ *versus* MS
							CFS ↑ *versus* controls
							Dep ↑ *versus* MS
							Dep ↑ *versus* controls
$CD20^+/CD23^+$	17 (7)	29 (23)	49 (18)	49 (16)	0·179		CFS ↓ *versus* MS
							CFS ↓ *versus* controls
NK cell subsets							
$CD16^+/CD3^-$	506 (14)	333 (11)	238 (13)	309 (12)	0·008*	CFS ↑ *versus* MS	
$CD56^+/CD25^+$	20 (18)	13 (23)	0 (22)	0 (23)	0·179		CFS ↑ *versus* MS
							CFS ↑ *versus* controls
							Dep ↑ *versus* MS
							Dep ↑ *versus* controls
$CD56^+/CD16^-$	66 (19)	116 (11)	78 (13)	58 (14)	0·115		Dep ↑ *versus* controls
$CD56^+/CD2^-$	80 (20)	52 (24)	39 (22)	42 (24)	0·123		CFS ↑ *versus* MS

Values are median absolute number of cells per μl. Numbers in parentheses are the number of samples tested. *Denotes a significant difference ($P < 0·01$) among the four comparison groups. Results of pairwise comparisons are shown for these subsets.

example, the number of $CD20^+$ B cells were calculated for each sample by first calculating the following cell number sums: $(CD20^+/CD5^- + CD20^+/CD5^+)$ $(CD20^+/CD29^- + CD20^+/CD29^+)$ $(CD20^+/CD25^- + CD20^+/CD25^+)$ and $(CD20^+/CD23^- + CD20^+/CD23+)$. For each sample, the average value of these four sums was the reported $CD20^+$ cell number value. Similar analyses were conducted for the $CD3^+$, $CD8^+$ and $CD56^+$ cells.

The gender make-up of the four comparison groups was tested for statistically significant differences using a χ^2 test on the 2×4 contingency table. As the age data were not normally distributed, differences in age between the four comparison groups were tested for significance using a Kruskal–Wallis analysis of variance (ANOVA) on rank test. Similarly, Kruskal–Wallis ANOVA on rank tests were used to detect differences between the comparison groups for each lymphocyte subset, as the cell number data also were not normally distributed. If a difference was found, pairwise comparisons using Dunn's method were performed to identify specific differences among the four groups. The SIGMASTAT (version 1·0, Jandel Scientific, San Rafael, CA, USA) software package was used for all statistical tests.

In this study we tested 81 different lymphocyte subsets for statistically significant differences. Because 81 different null hypotheses are being tested, it would be appropriate to lower the threshold *P*-value for statistical significance for each test, so that the overall *P*-value for all tests combined is 0·05. However, due to the exploratory nature of this study and the fact that many of the lymphocyte subsets are not independent of each other, a *P*-value correction for multiple comparisons is not recommended [36,37]. Because no correction was made for the total number of statistical tests, we chose to use a more conservative *P*-value threshold of 0·01 to determine statistical significance. We also identified group trends in the lymphocyte subsets where no significant differences were found. We report as trends only those groups that have cell numbers at least twice that of another group.

Results

Composition of study subject groups

Study subjects included 23 patients with CFS, 22 patients with MS, 24 patients with major depression, and 25 healthy control subjects. Members of each group were matched for age (within 5 years) and gender (Table 3). Duration of illness was also compared among the patient groups. Patients with CFS had a significantly longer duration of illness than patients with depression ($P = 0{\cdot}003$), but only six of 24 patients with depression provided the month and year of illness onset. From this we infer that most of the patients with depression could not recall specifically their illness onset. In contrast, all the patients with CFS remembered the onset of their illness because it was sudden.

Table 3. Demographics of study subject groups.

	No. of subjects	Age (years)[a]	Gender (% female)	Illness duration (years)[a]
CFS	23	38·6	73·9	3·9[b]
Depression	24	35·5	75·0	0·8[b,c]
MS	22	39·9	77·3	2·6[d]
Healthy controls	25	37·7	76·0	–
P-value	–	0·843	0·995	0·003

[a]Median values reported since non-parametric statistical testing was performed. [b]Denotes a statistically significant difference ($P < 0{\cdot}05$) between patients with CFS and patients with depression. [c]Value based on questionnaire responses from six patients. The other 18 patients did not provide this information. [d]Value based on questionnaire responses from 20 patients. The other two patients did not provide this information.

Total lymphocyte counts and major lymphocyte subsets

Comparing the four study subject groups, there were no statistically significant differences in absolute numbers of total lymphocytes, $CD2^+$ (T and most NK) cells, CD3+ T cells, $CD20^+$ B cells or $CD56^+$ NK cells (Table 1). No remarkable trends (absolute numbers of cells in one group at least twice the number in a comparison group) were detected among the groups for any of these major lymphocyte subsets.

T cell subsets

Comparing the four study groups, there was no trend towards any difference in total numbers of the CD4 helper T cell or CD8 suppressor/cytotoxic T cell subsets (data not shown). Patients with CFS tended to have increased numbers of activated cytotoxic T cells, as assessed by co-expression of CD8 with CD38 and HLA-DR (Table 2). Numbers of activated $CD8^+/CD38^+$ cells tended to be increased in patients with CFS compared to patients with MS, and numbers of CD8+/HLA-DR^+ cells tended to be higher in patients with CFS than in healthy controls. However, these differences did not achieve statistical significance. We did not detect any significant differences in $CD8^+/CD11b^+$ cells comparing the four study groups (data not shown). Patients with depression had a significantly lower number of resting T $(CD3^+/CD25^-)$ cells ($P = 0{\cdot}006$) in the peripheral blood compared to all other study groups.

B cell subsets

$CD20^+/CD5^+$ B cells were significantly elevated in patients with depression compared to all other groups ($P = 0{\cdot}004$). Activated $CD20^+/CD25^+$ B cells tended to be increased in patients with CFS and depression compared to patients with MS and healthy control subjects, but these differences were not statistically significant.

NK cell subsets

Of the various immunologic changes described in CFS patients, abnormalities in NK cell number and/or function have been reported most frequently. We therefore undertook an extensive analysis of NK cell subsets in CFS patients and the other study groups. As shown in Table 1, there were no statistically significant differences in absolute numbers of total $CD56^+$ NK cells. We also detected no significant differences or trends for the following NK cell subsets: $CD56^+/CD8^+$, $CD56^+/CD11b^+$, $CD56^+/CD38^+$ or $CD56^+/CD57^+$. However, the number of $CD16^+/CD3^-$ NK cells was significantly increased in patients with CFS compared to patients with MS ($P = 0{\cdot}008$). Both patients with CFS and depression tended to have increased numbers of $CD25^+$ NK cells compared to patients with MS and healthy control subjects, although the differences did not achieve statistical significance.

Discussion

We compared lymphocyte subsets of patients with CFS to those of patients with major depression and MS as well as those of healthy control subjects, with the goal of identifying immunophenotypic differences in patients with CFS compared to two patient groups with fatiguing illnesses – major depression and MS – and to healthy control subjects.

We found a trend ($P = 0{\cdot}057$–$0{\cdot}082$) towards increased numbers of $CD8^+/HLA\text{-}DR^+$ and $CD8^+/CD38^+$ in CFS patients compared to patients with MS or control subjects. It is possible that inclusion of larger numbers of study subjects would have allowed this trend to achieve statistical significance. This is in keeping with the reports of Klimas *et al.* [38], Landay *et al.* [39], Hassan *et al.* [40] and Peakman *et al.* [41], but not with several other reports [42–44].

Like Straus *et al.* [42] and Natelson *et al.* [43], but unlike Landay *et al.* [39], we did not detect any trend towards depressed numbers of $CD8^+/CD11b^+$ suppressor T cells in patients with CFS compared to control subjects. Of note, in the study of Landay *et al.* [39] the increased percentages of $CD8^+/HLA\text{-}DR^+$ and $CD8^+/CD38^+$ cells and depressed proportion of $CD8^+/CD11b^+$ cells were seen only in the most severely affected patients with CFS (those who were functioning at less than 25% of their normal daily activity).

While reduced NK cell function has been found repeatedly in patients with CFS [45,46], phenotypic findings have been inconsistent: low [45,47–49], normal [39,42,43] or even increased [38,41,50] percentages or absolute numbers of NK cells have been found in the peripheral blood of these patients. Although we did not detect any significant difference in the number of total NK cells in CFS patients, patients with CFS had significantly increased numbers of the $CD16^+/CD3^-$ NK cell subset in the peripheral blood compared to patients with MS. In contrast to Morrison *et al.* [50], we did not find an increased number of $CD16^-$ ($CD56^{bright}$) NK cells or a decreased proportion of $CD16^+$ NK cells in patients with CFS. Resting $CD56^{dim}$ $CD16^+$ NK cells do not express detectable levels of CD25 on the cell surface [51,52] but can express this antigen after *in vitro* activation [53,54]. $CD25^+$ NK cells were observed in patients with CFS and depression, but were not detected in the blood of patients with MS or healthy subjects. Analysis of larger numbers of patients and control subjects will be required to determine if this trend is significant. Moreover, additional studies are needed to determine whether NK cell function is activated *in vivo* in CFS patients with higher numbers of $CD25^+$ NK cells.

In contrast to Klimas *et al.* [38] and Tirelli *et al.* [48], but in accordance with other investigators [39,42,47,49], we did not find increased numbers of either total B cells or of $CD5^+$ B cells in patients with CFS compared to healthy subjects. Neither Klimas [38] nor Tirelli [48] evaluated the patients with CFS carefully for concomitant major depression. We have found that major depression may be characterized by higher numbers of $CD5^+$ B cells (see below). We did find that patients with CFS tended to have increased numbers of activated B ($CD20^+/CD25^+$) cells compared to patients with MS and healthy control subjects, although this trend was not statistically significant ($P = 0{\cdot}044$).

In comparison to the other groups, patients with major depression had significantly higher numbers of $CD20^+/CD5^+$ B cells. We are not aware that this has been reported previously. $CD5^+$ (or B-1) B cells are a distinct subset of mature B cells that constitutes approximately 1–7% of peripheral blood leucocytes (PBL) in healthy adult humans [55,56]. The immunoglobulin genes of $CD5^+$ B cells usually do not show evidence of somatic hypermutation and $CD5^+$ B cells tend to produce low affinity IgM autoantibodies. Maes *et al.* [57,58] found increased levels of total B cells in depressed patients, whereas others have reported no difference in total B cell numbers compared to control subjects [59–63]. However, none of these studies describes an analysis of $CD5^+$ B cells.

We observed a highly statistically significant decrease in resting ($CD25^-$) T cells in patients with depression, compared to all other subject groups; this was accompanied by a non-significant trend ($P = 0{\cdot}061$) toward higher numbers of activated $CD25^+$ T cells. Maes *et al.* [58,64] have reported increased numbers of $CD25^+$ and $HLA\text{-}DR^+$ lymphocytes in patients with depression. They subsequently identified these $CD2^+/HLA\text{-}DR^+$ and $CD7^+/CD25^+$ lymphocytes as activated 'T cells' [65]. However, because both T cells and NK cells express CD2 and CD7, it is possible that these $CD2^+/CD7^+/CD25^+/HLA\text{-}DR^+$ lymphocytes comprise $CD16^-$ NK cells as well as activated T cells. If so, the results of Maes *et al.* would be largely in agreement with ours with respect to depressed patients. In contrast to some previous studies [43,66–68], we did not observe any trend toward increased numbers of $CD25^+$ T cells and $CD8^+/CD11b^+$ T cells in patients with MS compared to control subjects, nor any trend toward decreased numbers of $CD8^+/HLA\text{-}DR^+$ T cells. However, the

most consistent abnormalities that have been reported in MS involve the $CD4^+/CD45RA^+$ T cell subset.

We have identified several differences in T, B and NK cell subsets in patients with CFS and depression that were not present in patients with MS. Some of these changes were shared by both patients with CFS and patients with depression, whereas others were different. We cannot determine whether the alterations in lymphocyte subsets that we observed are directly involved in the pathophysiology of these disorders, or whether they are secondary effects. Future studies will be required to distinguish between these possibilities.

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